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Transfer of condensed viral DNA into eukaryotic cells using proteoliposomes

(Liposomes; influenza virus glycoprotein; DNA condensation; transgenosis; transfection; adenovirus; simian virus 40)

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SUMMARY

High-molecular-weight viral DNAs have been packed into proteoliposomes prepared by reverse-phase evaporation followed by phospholipid membrane targeting by influenza virus glycoprotein bound to hydrophobic 'anchors'. DNA has been encapsulated in the form of spermine condensates — toroidal structures sized approx. 0.1 μm, resistant to ultrasound. The efficiency of entrapping into liposomes reached 30% for condensed DNA of M_r up to 3×10^7. Specific infectivity of simian virus 40 DNA and simian adenovirus DNA packed into such proteoliposomes was 50- to 100-fold higher than that shown by free DNA preparations under Ca · phosphate-precipitation conditions.

INTRODUCTION

Transgenosis, or transfection, i.e., transfer of genetic material into the cell is a limiting factor for many biological experiments. Great difficulties are involved with transfection of eukaryotic cells by high-M_r viral DNA, because the available methods, starting with the first successful experiment of Szybalska and Szybalski (1962), are either of low efficiency (Graham and Van der Eb, 1973) or technically complicated (Diakumakos and Gershey, 1977). Artificial phospholipid vesicles are ideal DNA carriers in many respects (Gregoriadis and Allison, 1980): they are nontoxic and biodegradable, they protect the trapped DNA from DNase, and provide transport of nucleic acids into the cells and cellular organelles. However, liposomal transfer has yet to be investigated and its optimal characteristics have to be determined. The employment of liposomes for the

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Abbreviations: *A*, absorbance; DMSO, dimethyl sulfoxide; DTAB, dodecyl trimethylammonium bromide; GMK, green monkey kidney; HA, hemagglutinating activiity or hemagglutinin; HL, hemolysin, hemolysing activity; PA, polyacrylamide; PFU, plaque-forming units; RPE, reverse-phase evaporation; SAd, simian adenovirus; SDS, sodium dodecyl sulfate; SV, simian virus.

transport of high-M_r DNA into the cells entails additional problems, since packaging of this DNA is hindered by its large size and sensitivity to ultrasound required for the preparation of liposomes by the RPE method (RPE liposomes). In this study we have carried out the condensation of DNA which practically abolished the size dependency for DNA and allowed its highly efficient encapsulation into liposomes prepared by the RPE method. Previously, linear DNA with an M_r of no more than 0.6×10^6 would be encapsulated into such vesicles (Wong et al., 1980), whereas in our experiments DNAs with M_rs up to 3×10^7 were packaged.

For optimization of DNA transfer into the cells, liposomes carrying influenza virus glycoproteins on their surface have been used. The targeting of preformed liposomal membrane was carried out by proteins chemically modified with the help of palmitoyl chloride, which functions as a hydrophobic anchor. Specific infectivity of viral DNA transferred by RPE liposomes into the cells was thereby significantly increased. Some of the preliminary results have been reported by Glushakova et al. (1985).

MATERIALS AND METHODS

(a) Biochemicals

Spermine · 4 HCl and DNase II were from Calbiochem; cholesterol and DTAB were from Sigma; Ficoll-400 was from Pharmacia; other reagents were from Reakhim. Phosphatidylcholine was isolated from chicken egg yolks by the method described by Dubichev and Glushkova (1985); the purity of the phospholipid preparation was tested according to the method of Svetashev and Vaskovski (1972).

(b) DNA

DNA from phage λ and adenovirus SAd7 was isolated using the the procedure of Maniatis et al. (1982) and Bello and Ginsberg (1969), respectively. SV40 DNA was isolated from plasmid pSGI carrying a complete genome of this virus, with subsequent ligation and electrophoresis in agarose gels using conventional techniques (Maniatis et al., 1982). Condensation of viral DNA by spermine and subsequent analysis were carried out as described by Gosule and Schellman (1976) and Chattoray et al. (1978). DNA was analyzed by electrophoresis in 0.7% agarose gel by the technique of Helling et al. (1974).

^{32}P-labeled DNA was prepared by nick-translation using a modification of the method of Maniatis et al. (1975). Special care was taken in completely removing DNase from all solvents. The reaction was conducted in the medium with two [^{32}P]nucleotides for 30 min at 12°C. Specific activity of DNA equalled an average of 2×10^5 cpm/μg of DNA.

(c) Liposome preparation

DNA was encapsulated into RPE liposomes according to the procedure of Fraley et al. (1980; 1981) with the following modifications. 10 μM of phosphatidylcholine and 3.3 μM of cholesterol in 0.5 ml of ether were taken. Aqueous phase (20 mM Tris · HCl pH 7.6, 25 mM NaCl) contained 1 to 10 μg of native or condensed DNA in 160 μl. Untrapped DNA was separated by flotation in Ficoll. For nuclease treatment of liposomal preparation DNase II (final concentration 100 μg/ml) was used, the reaction was conducted at 37°C for 60 min. For bioassays DNA was encapsulated into liposomes and subsequently targeted according to the technique of Shen et al. (1982).

Influenza virus A/FPY/Rostock/H7/NI was used. It was grown in chick embryos, purified and concentrated by the method of Kingsbury (1966). Influenza virus glycoproteins were isolated using the cationic detergent DTAB that can selectively solubilize the viral glycoproteins (Glushakova et al., 1985b). The isolated proteins were analyzed by SDS–PA gel electrophoresis according to the procedure of Laemmli (1970). Protein concentration was measured by the method of Lowry et al. (1951). The HA was chemically modified by palmitoyl chloride as described by Torchilin et al. (1980). Hemagglutinating and HL activities of glycoproteins in free state and in proteoliposomal preparations were assessed according to Fazekas et al. (1966) and Sato et al. (1983), respectively.

(d) Transfection

Infectivity of viral DNA was tested in primarily trypsinized GMK cell cultures. The infection was

carried out by the Ca · phosphate precipitation method (Van der Eb and Graham, 1980) or by liposomal techniques (Fraley et al., 1981; Mertz and Berg, 1974) with some modifications. Prior to the exposure to DNA-containing material, the cells in each 60-mm dish containing 10^6 cells were activated by 20% glycerol (4 min). The exposure of cells to liposomes or Ca · phosphate precipitate lasted 60 min at 37°C with periodic gentle shaking of the flasks followed by agar layering (Mertz and Berg, 1974). The results were recorded on days 18–20 postinfection by counting the number of plaques.

RESULTS

(a) Encapsulation of condensed viral DNA into RPE liposomes

Only large unilamellar vesicles of all the liposomes can efficiently encapsulate large linear molecules, such as DNA (Straus et al., 1981). Liposomes prepared by the RPE method can entrap up to 65% of water-soluble material, which is more than other large liposomes can (Szoka and Papahadjopoulos, 1978). The incorporation of circular superhelical SV40 DNA has been reported (Fraley et al., 1980; 1981). However, the preparation of these liposomes requires intensive ultrasonic treatment, which leads, as we have shown, to complete destruction of linear viral DNAs (of adenovirus SAd7 and of phage λ) with M_rs of 22×10^6 and 30×10^6, respectively (Glushakova et al., 1983).

(1) Spermine-induced DNA condensation

For efficient incorporation of linear high-M_r DNA into vesicles we have attempted to condense these nucleic acids, i.e., to prepare compact DNA forms resistant to ultrasound and appropriate in size to fit into RPE liposomes. To this end we applied spermine, for the first time used by Szybalska and Szybalski (1962) for transfection of human cells.

Electron microscopy of condensed DNA structures produced in water–salt–spermine solutions has shown that under optimal condensation conditions, linear λ DNA was turned into a compact toroidal structure (Fig. 1). Spermine concentration needed for torus formation was primarily dependent on ionic strength of the medium. Thus, for example, in 1 mM NaCl, 1 mM Na · cacodylate, 10^{-2} mM EDTA, pH 6.45, condensation of DNA is initiated at spermine concentration of 24 μM 24 (Fig. 1A). In addition to toroid structures, uncondensed DNA strands can also be seen. At spermine concentration of 30 μM virtually all the DNA assumes a compact toroidal form (Fig. 1B). The rise of spermine concentration to 36 μM will result in aggregation of tori (not

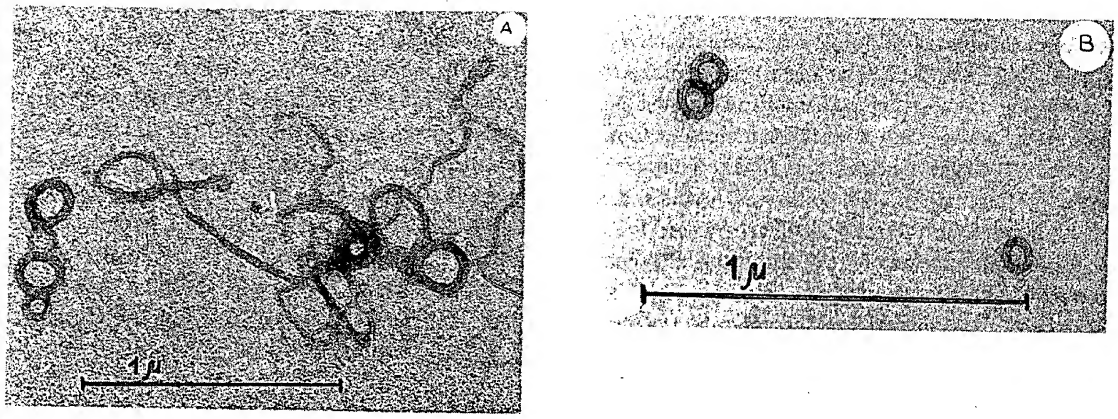

Fig. 1. DNA condensation by spermine in buffer with low ionic strength. (A) Spermine concentration 24 μM. (B) Spermine concentration 30 μM. The bar corresponds to 1 μm. Condensation of viral DNA by spermine and subsequent analysis were carried out as described by Gosule and Schellman (1976) and Chattoray et al. (1978). Briefly, viral DNA (λ or SAd7) was dissolved in low-ionic-strength buffer (1 mM NaCl, 1 mM Na · cacodilate, 10 mM EDTA, pH 6.45) containing indicated spermine concentrations. After a 20-min incubation at 20°C the mixtures were prepared for electron-microscopic analysis. DNA preparations were applied to collodion grids and shadowed with Pt : Pd (1 : 4) at a 10° angle.

shown). A similar picture has been observed in a medium with higher ionic strength: in 25 mM NaCl the tori were produced at 65 μM of spermine, and in 50 mM NaCl at 300 μM of spermine. DNA condensates were rather stable: incubation for 60 min at 37°C or for 16 h at 4°C failed to cause decondensation. However, the tori were transformed into loose structures with increasing ionic strength. Average torus diameter was equal to approx. 0.1 μm, which is smaller than the average diameter of RPE liposomes (0.3 μM) (Szoka and Papahadjopoulos, 1978).

(2). Resistance to ultrasound

The ability of spermine to enhance DNA resistance to ultrasound was tested by electrophoretic analysis in agarose gels of the samples that underwent condensation followed by ultrasonic treatment. It should be noted that the electrophoretic buffer had a higher ionic strength than the medium for DNA condensation, which resulted in partial removal of spermine from the DNA during application of the sample on the gel.

As seen in Fig. 2, in the presence of 25 mM NaCl, spermine at concentrations of 65 μM and higher protected DNA from ultrasonic destruction. In 145 mM NaCl complete destruction of DNA was observed at spermine concentrations up to 400 μM (higher concentrations have not been tested). It is noteworthy, that DNA preparations protected by spermine from ultrasonic damage had a somewhat lower electrophoretic mobility than control DNA (Fig. 2, lanes J and L), which can be accounted for by possible residual neutralization of some DNA charges by undissociated spermine molecules. Spermine condensation is reversible, for the increase in the ionic strength in the condensed nucleic acid preparation from 25 mM NaCl to 145 mM and subsequent sonication of the preparation led to complete DNA destruction (Fig. 2, lane M). This confirms electron microscopic observations on decondensation of tori in the medium with increased ionic strength.

(3) Efficiency of encapsulation

Using [^{32}P]DNA we have determined the efficiency of encapsulation of spermine condensates of viral DNA into liposomes. To this end, [^{32}P]DNA of λ or SAd7 were condensed by spermine, incorporated into liposomes, and the radioactivity of loaded vesicles purified by flotation in Ficoll was counted (Table I). It is seen that the entrapment efficiency into liposomes for uncondensed low-M_r DNA was 45%, and that of high-M_r condensed tori was 30%. The pattern of label distribution in the Ficoll gradient fractions after flotation was essentially unchanged when the liposomes were pretreated with DNase (not shown).

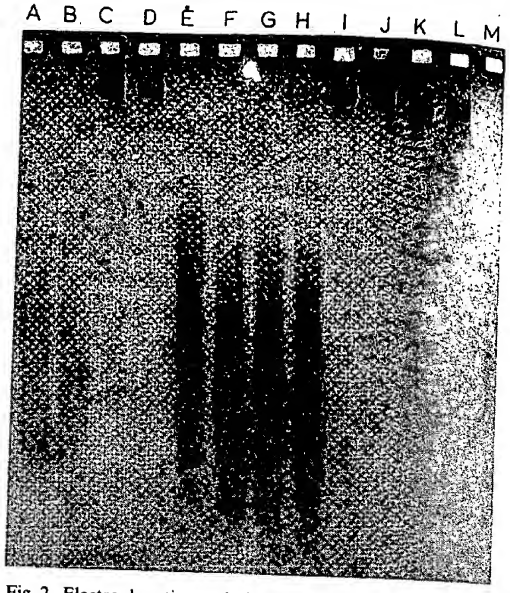

Fig. 2. Electrophoretic analysis of viral DNA preparations treated with spermine at indicated ionic strengths and exposed to ultrasound. Lanes A–D, buffers of low ionic strength (25 mM NaCl), with spermine concentrations: A, 30 μM; B, 60 μM; C, 65 μM; D, 80 μM. Lanes E–H, buffers with higher ionic strength (145 mM NaCl), with spermine concentrations: E, 100 μM; F, 200 μM; G, 300 μM; H, 400 μM. Lanes: I, control phage DNA preparation; J, phage λ DNA in the buffer with low ionic strength and 80 μM spermine; K, control SAd7 DNA preparation; L, SAd7 DNA in the buffer with low ionic strength and 80 μM spermine; M, SAd7 DNA condensed in the medium with low ionic strength and sonicated immediately after an increase of NaCl concentration in the medium to 145 mM. In each case, 4 μg of DNA were sonicated in 160 μl of the buffer at specified spermine concentrations for 15 s in a bath-type disintegrator.

(b) Preparation of proteoliposomes

The interaction of liposomes with cells is known to be enhanced in the presence of some ligands coupled to vesicular membrane (Szoka et al., 1981; Torchilin and Klibanov, 1981). We have chosen

TABLE I

Efficiency of encapsulation of condensed and uncondensed viral [^{32}P]DNA into RPE liposomes determined by liposome flotation in Ficoll

Preparation[a]	Radioactivity of liposomal preparation (cpm)		Ratio of radioactivities of liposomal preparation after and before Ficoll flotation (%)
	Before Ficoll flotation[d]	After Ficoll flotation[d]	
Liposomes loaded with uncondensed viral [^{32}P]DNA (λ or SAd7)[b]	345 650	155 540	45
Liposomes loaded with viral [^{32}P]DNA (λ or SAd7) condensed by spermine[c]	176 700	54 700	31

[a] Condensed and uncondensed viral [^{32}P]DNAs were encapsulated in RPE-liposomes as described in MATERIALS AND METHODS, section **c**.

[b] Liposome-encapsulated uncondensed [^{32}P]DNA had a low M_r because of destruction resulting from sonication during liposome preparation.

[c] Liposome-encapsulated viral [^{32}P]DNA condensed with spermine had a high M_r.

[d] Liposomes were purified from free [^{32}P]DNA by flotation in Ficoll gradient according to the procedure of Glushakova et al. (1985b).

influenza virus glycoproteins as ligands for preparation of targeted vesicles that would simulate first stages of viral infection by interacting with cell receptors.

For selective solubilization of viral glycoproteins we have used cationic detergent DTAB, which, unlike its analog cetavlon that was previously used for these purposes, can be easily removed from protein solutions by dialysis. Selective solubilization of viral glycoproteins requires a detergent concentration of 0.15–0.20%. The protein yield equalled an average of 50–60% of the theoretically expected value. Comparative characterization of HA and HL activities in the preparation of glycoproteins and in concentrated virus suspension (Table II) has shown that both activities are preserved in the HA isolated by DTAB, HA activity being increased and HL activity somewhat decreased (HA accounts for 20% of the viral protein).

Before incorporation of viral HAs into liposomes, palmitic acid residues, the so-called 'hydrophobic anchors' having a high affinity to the lipid bilayer, were covalently linked to the basic amino acids (Torchilin et al., 1980). All attempts to couple unmodified glycoproteins to the membrane of preformed liposomes were unsuccessful.

Coupling of the hydrophobically modified HAs with liposomal lipids was performed in the presence of diethyl ether or 0.15% deoxycholate, which to some extent destabilizes the lipid bilayer. Subsequent dialysis removes the ether and the detergent from the liposomes, thereby enhancing and stabilizing the hydrophobic interactions of the modified proteins with the liposomal membrane (Shen et al., 1982).

We have previously assessed the effect of sodium deoxycholate and ether on HA activities in the glycoprotein preparations. The detergent did not reduce the protein's activities, while the ether decreased HA activity up to 800-fold, and HL activity three-fold.

Coupling of modified glycoproteins to liposomal membranes was carried out at a protein : lipid ratio of 1:10. It follows from the data presented in Table III that, after the interaction with viral glycoproteins, the liposomes acquired the ability to agglutinate and lyse chicken red blood cells. Control liposomal preparations without coupled proteins did not possess HL activity, but displayed an unexpected HA activity amounting to 2 HA units (not shown).

The percentage of glycoproteins coupled with liposomes was dependent on the initial protein : lipid ratio taken in the experiment (Table IV). The absence of an expected increase in the percentage of linked

TABLE II

Comparative characterization of hemagglutinating (HA) and hemolysing (HL) activities of DTAB-isolated glycoproteins and concentrated viral preparations

	Glycoproteins[a] or virus[b]	Activity
HA activity[c]	Glycoprotein 20 μg of protein	262 100 HA units
	Virus 20 μg of protein	32 700 HA units which is equivalent to 163 500 HA units per 20 μg of virus HA
HL activity[d]	Glycoproteins 20 μg/ml	4.27 *A* units
	Virus 20 μg/ml of protein	3.84 *A* units which is equivalent to 19.20 *A* units per 20 μg/ml of virus HA

[a] Glycoproteins were prepared by treatment of concentrated virus suspension (1 mg/ml of protein) in 145 mM NaCl, 5 mM Na_2HPO_4, 0.2% DTAB for 60 min at room temperature; the suspension was then layered on 2 vols. of 10% sucrose on the same buffer and centrifuged in an SW 50-1 rotor for 90 min at 35 000 rev./min, 4 °C. Glycoprotein-containing supernatant fluid was intensively dialysed against distilled water pH 7.5–8.0, concentrated to 1.5 mg/ml, and HL activity was determined.

[b] Virus was prepared as described in MATERIALS AND METHODS, section c.

[c] HA activity was determined according to Fazekas et al. (1983).

[d] HL activity was determined according to Sato et al. (1983) and expressed as 520 nm *A* of a hemoglobin solution released by red blood cell lysis caused by the virus and glycoproteins in acidic medium, pH 5.2.

protein at a protein : lipid ratio of 1 : 2 can be accounted for by the fact that these liposomes tend to aggregate. As a result some proteoliposomes remain on the bottom of the test tube during flotation and are not taken into account. This suggestion has been verified in the experiment on fractionation in Ficoll gradient (not shown). Since protein binding with RPE-liposomes required a modification of the original method, we have determined the efficiency of entrapment of spermine-condensed [^{32}P]DNA into such targeted vesicles. It proved to be lower than with untargeted liposomes (30%, as mentioned above) and equalled 12.3%. These findings conform with the data of the authors who coupled modified proteins with RPE-liposomes (Sheb et al., 1982).

TABLE III

HA and HL activities of glycoprotein-reconstructed liposomal preparations

Experiment No.	Preparation[a]	HA activity[b] (units)	HL activity[c] (*A* units)
1	Test	512	1.3
	Control	64	0
2	Test	16	1.9
	Control	2	0.2

[a] Equal volumes of liposomal preparations with equal lipid content were tested (lipid content was determined by [^{3}H]-cholesterol incorporated into liposomal membrane).

[b] Much lower values of HA activity in the 2nd experiment can be explained by high inhibitory effect of the ether on the protein; standardization of the amount of the ether in liposomal suspension at the time of protein addition involves methodological problems. For assay method see Table II, footnote c.

[c] For methods see Table II, footnote a.

Both liposomes and proteoliposomes were able to transfer their content into eukaryotic cells as evident from experiments with labeled [^{32}P]DNA (Table V). It can be seen from the data presented that the percentage of the label transferred into the cells by liposomes was very low in all cases. Because radioactive compounds encapsulated in liposomes do not permit the distinction between intracellular delivery of liposome content and the adsorption of vesicles to the cell surface, we have assessed biological activity of liposome-mediated transfer of DNA.

(c) Transfection of green monkey cell culture by viral DNA packaged into liposomes

To assess the efficiency of liposomal transfer of biologically active viral DNA into the GMK cells, we have determined the infectious titers (PFU/μg of DNA) of SV40 DNA and SAd7 DNA packed into RPE liposomes. The results were compared with the values obtained in parallel experiments for transfection of the same GMK cell culture by DNA preparations under the conditions of Ca · phosphate-precipitation. The results summarized in Table VI show that liposomes provide a more efficient transfer of viral DNA into the cells (of both linear SAd7 DNA and circular SV40 DNA) than Ca · phosphate precipitate; for SV40 DNA the use of standard lipo-

TABLE IV

Percentage of protein bound to liposomes determined by flotation in Ficoll

Protein/liposome ratio [a]	Radioactivity of proteoliposomal preparation (cpm)		% of protein incorporation into proteoliposomes [d]
	Before flotation [b]	After flotation [c]	
1:10	2400	900	37.5
1:5	7200	4350	60.4
1:2	15200	8000	53.6

[a] Weight ratio of added protein to lipid of preformed liposomes.

[b] The amount of protein bound to liposomes during reconstitution was determined with the use of glycoproteins to which [^{3}H]palmitic acid residues were previously linked. H-succinimidyl-32, 3-[^{3}H]propionate in a minimal volume of DMSO was mixed with glycoproteins and kept for several hours at room temperature; the unbound label was then removed by 6-h dialysis (three changes of 1000-fold volumes). Labeled proteins were then incubated with liposomes under conditions specified in RESULTS, section **b**. Numbers represent total radioactivity (proteoliposomes plus unbound labeled proteins).

[c] After flotation, the proteoliposome preparation was suspended in the initial volume of the buffer, and radioactivity was measured in aliquots equal to those mentioned in footnote b.

[d] Calculated as the part of total radioactivity associated with the fraction of proteoliposomes floating to the top of the Ficoll gradient. See footnote d in Table I.

TABLE V

Transfer of viral DNA into GMK cell culture by Ca · phosphate precipitation method and liposomal technique

Method of transfer [a]	Radioactivity applied to cells (cpm)	Radioactivity bound to cells [b]	
		cpm	% of total
Ca · phosphate-precipitate	469280	220	0.04
RPE liposomes	68780	390	0.54
Proteoliposomes '1:10'	120870	1400	1.16

[a] Ca · phosphate precipitate of [^{32}P]DNA, preparations of liposomes and proteoliposomes loaded with the same [^{32}P]DNA were prepared according to Mertz and Berg (1974), Fraley et al. (1980) and Szoka et al. (1981), respectively. The design of all the experiments included the following stages: the semiconfluent monolayers (approx. 5×10^6 cells per 15-cm^2 flask) of GMK cells were thoroughly washed with buffer-salt solution from serum-containing culture medium; they were treated for 4 min with 4 ml of 20% glycerol. Liposomal or proteoliposomal [^{32}P]DNA preparations or [^{32}P]DNA Ca · phosphate-precipitate preparations were incubated with cell monolayer for 30 min at 37°C with subsequent addition of 5 ml of the medium to the cells followed by a 6-h incubation.

[b] In the case of liposomes, the vesicles which did not penetrate into the cells were desorbed by competitive method with excess of 'empty' liposomes and in the case of DNA Ca · phosphate material adsorbed on the external side of cytoplasmic membrane was desorbed by buffer with 5 mM EDTA. Radioactivity bound with cells after their removal from the glass by Versene solution was counted.

somes yielded a specific infectivity 34 times as high as in the Ca · phosphate-precipitation technique. The use of proteoliposomes (1 : 5 type) provided a specific infectivity of DNA that was 55 times as high. For SAd7 DNA the specific infectivity was increased approx. three times (as compared to the Ca · phosphate-precipitation technique) in the case of standard liposomes, and 107 times in the case of proteoliposomes (1 : 5 type). The effect of targeting of vesicular membrane by viral glycoproteins was especially marked for adenoviral DNA (infectivity increased 38-fold).

Specific infectivity of SAd7 DNA in proteoliposomes of the 1 : 2 type was one-fourth of that in proteoliposomes of the 1 : 5 type. The aggregation of the first type of liposomes seems to be the most likely explanation: as a result of the aggregation they float poorly in Ficoll and are scarcely absorbed by the cells via endocytosis (Schwendener et al., 1984; see also RESULTS, section **b**). Proteoliposomes of the 1 : 5 type, which yielded a higher specific infectivity than type 1 : 10 proteoliposomes (not shown) seem to be the optimal tool for the transfer of exogenous substances into the cell. It should be noted that proteoliposomes which were not subjected to fractionation in Ficoll gradient had a lower specific infectivity than the floated vesicles.

TABLE VI

Infectivity of simian adenovirus SAd7 DNA and virus SV40 DNA

Preparation	Infectivity, PFU/μg of incorporated DNA[d]		Specific infectivity of liposomes; Ca · precipitate ratio		Specific infectivity liposome/proteoliposome[e]	
	SV40	SAd7	SV40	SAd7	SV40	SAd7
Liposomes[a]						
With flotation	612.4	7.6	34.5	2.8	—	—
Without flotation	332.2	—	18.5	—	—	—
Proteoliposomes 1:2[b]						
With flotation	—	66.6	—	24.7	—	8.8
Without flotation	—	—	—	—	—	—
DNA Ca · phosphate-precipitate[c]	18.0	2.6	—	—	—	—

[a] Liposomes loaded with condensed viral DNAs were prepared according to Fraley et al. (1980). For details see MATERIALS AND METHODS, section c.

[b] Protein-to-lipid ratio. See footnote [a] to Table IV.

[c] Ca · phosphate precipitate of viral DNAs was prepared according to Mertz and Berg (1974).

[d] Infectivity of viral DNAs incorporated into liposomes and proteoliposomes or of Ca · phosphate precipitates was determined as described in MATERIAL AND METHODS, section d. Specific infectivity calculations were made per 1 μg of DNA included into vesicles or per 1 μg of input DNA (in case of Ca · phosphate precipitates).

[e] The ratio was calculated between the specific infectivities of similarly purified liposome and proteoliposome preparations.

DISCUSSION

The transfer of DNA into eukaryotic cells followed by the investigation of their expression has been widely used for the studies of the structure and function of genetic material. Several methods of the transfer of nucleic acids into the cells have been proposed: precipitation with Ca · phosphate (Graham and Van der Eb, 1973), microinjection into the cell nucleus (Diacumakos and Gershey, 1977), the use of DEAE-dextran (Lewis et al., 1980), of red blood cell ghosts (Ihler et al., 1973) and some others. Recently, phospholipid liposomal vesicles have been employed for this purpose (Gregoriadis and Allison, 1980). The main advantages of liposomes are: DNA protection against nucleases, the possibility to work with DNA without carriers, multiplicity of targeted cells, gentle treatment of the cell, the possibility of simultaneous transfer of DNA into many cells, standard experimental procedure owing to good preservation of liposomal preparations, technical simplicity of the experiment, the possibility to use liposomal vesicles in the in vivo experiments. At the same time, the types of liposomes to be used for packaging of high-M_r linear DNA have to meet most stringent requirements. They should be large, preferably unilamellar vesicles, with large internal volume, highly efficient in incorporating water-soluble material. RPE liposomes are the best in this respect, however, their preparation requires an ultrasonic treatment and only small linear DNA with M_r below 6×10^5 can be incorporated undamaged into such liposomes (Wong et al., 1980). Maximal size of a DNA packed into RPE-liposomes reached M_r 3×10^6, but that was a circular superhelical DNA (Fraley et al., 1980). Most experiments dealing with packaging of DNA into liposomes have been carried out with the help of other methods (Straus et al., 1981), but the incorporation efficiency was very low.

All these problems can be overcome by using pre-condensed DNA preparations. The idea of DNA condensation in terms of probable increase in efficiency of its incorporation into liposomes has been discussed by Fraley et al. (1980). We have shown, however, that this procedure can protect the DNA against ultrasonic destruction. Therefore, the RPE method can be used for highly efficient encapsulation of linear DNA, probably of rather high M_r without any damage to the biological activity of

DNA. Our data have demonstrated spermine-induced production of stable toroidal structures efficiently entrapped by RPE-liposomes. Considerable decrease of DNA concentration in the course of its condensation by spermine results in the production of tori containing a minimal amount of DNA molecules and thereby increases the percentage of DNA-containing liposomes (Kislina et al., 1985). The efficiency of incorporation of condensed DNA into RPE liposomes is higher, practically by a factor of 10^3, than that of encapsulation of native adenoviral DNA into large unilamellar liposomes (Straus et al., 1981).

The optimization of the transfer of packaged genetic material into the cells has been provided by the use of liposomes coupled with influenza virus glycoproteins. The recently demonstrated pH-induced membrane-fusing activity of influenza virus HA (Maeda and Ohnishi, 1975), as well as its thoroughly investigated receptor function (Wilson et al., 1981) have made this protein very attractive in terms of application in liposomal techniques of the transfer of biologically active substances into eukaryotic cells (White et al., 1982). Glycoprotein introduced into the membrane of the liposome loaded with the substance in question practically makes of it a 'container' that will efficiently transfer its content undegraded into the cell cytoplasm by endocytosis. In other words, such proteoliposomes will take the same path in the cells as influenza virions, which liberate their nucleocapsids into the cytoplasm after receptor interaction with the cellular surface and the passage through the endosomal apparatus of the cell. The fusion activity can be assessed by hemolysis of red blood cells interacting with the virus in an acidic medium.

We have reconstituted the preformed liposomes by influenza virus glycoproteins linked with palmitic acid residues which functioned as hydrophobic 'anchors'. The method has previously been used for coupling of immunoglobulins with liposomes (Shen et al., 1982). Detergent dialysis successfully used for the preparation of virosomes (Huang et al., 1980) is not suitable for incorporation of DNA into liposomes.

The proteoliposomes described in this paper exhibited virus-specific HA and HL activities. The disproportional decrease in HA activity in our liposomal preparations as compared to their HL activity, can be explained by the above-mentioned HA inhibition from ether treatment as well as by unusual orientation of HA molecules after their 'anchoring' into the liposomal membrane, which may reduce the efficiency of the interaction of protein with cellular receptors.

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REFERENCES

Bello, L.J. and Ginsberg, H.S.: Relationship between deoxyribonucleic acid-like ribonucleic acid synthesis and inhibition of host protein synthesis in type 5 adenovirus-infected cell. J. Virol. 3 (1969) 106–119.

Chattoray, D.K., Gosule, L.C. and Schellman, J.A.: DNA condensation with polyamines, II. Electron microscopic studies. J. Mol. Biol. 121 (1978) 327–337.

Diacumakos, E.G. and Gershey, E.L.: Uncoating and gene expression of simian virus 40 in CV-1 cell nuclei inoculated by microinjection, J. Virol. 24 (1977) 903–906.

Dubichev, A.G. and Glushakova, S.E.: A modified procedure for isolation of phosphatidyl choline and phosphatidyl ethanolamine using high pressure liquid chromatography. Vopr. Med. Khim. 4 (1985) 128–131 (in Russian).

Fazekas de St. Groth, S. and Webster, R.G.: Disquisitions on original antigenic sin, I. Evidence in man. J. Exp. Med. 124 (1966) 331–345.

Fraley, R.T., Subramani, S., Berg, P. and Papahadjopoulos, D.: Introduction of liposome-encapsulated SV40 DNA into cells. J. Biol. Chem. 255 (1980) 10431–10435.

Fraley, R., Straubinger, R.M., Rule, G., Springer, L. and Papahadjopoulos, D.: Liposome-mediated delivery of deoxyribonucleic acid into cells: enhanced efficiency of delivery related to lipid composition and incubation conditions. Biochemistry 20 (1981) 6978–6987.

Glushakova, S.E., Kislina, O.S., Naroditsky, B.S., Grigoriev, V.B., Manikin, A.A. and Tikchonenko, T.I.: Encapsulation of high molecular weight viral DNAs into liposomes using the condensing agents. Mol. Genet. Mikrobiol. Virol. 6 (1983) 31–37 (in Russian).

Glushakova, S.E., Grodnitskaya, N.A., Naroditsky, B.S., Kiskina, O.S., Tikchonenko, T.I., Melnikov, S.D. and Ghendon, Yu.Z.: Optimal transfer and incorporation of viral DNA into cells via liposomes connected with influenza viral glycoproteins. Mol. Genet. Mikrobiol. Virol. 7 (1985a) 32–36 (in Russian).

Glushakova, S.E., Naroditsky, B.S., Tikchonenko, T.I., Klibanov, A.L. and Torchilin, V.P.: Influenza viral glycoproteins isolation using cationic detergent dodecylmethylammonium bromide and its subsequent integration into liposomal membrane. Mol. Genet. Mikrobiol. Virol. 4 (1985b) 39–44 (in Russian).

Gosule, L.C. and Schellman, J.A.: Compact form of DNA induced by spermidine. Nature 259 (1976) 333.

Graham, F.L. and Van der Eb, A.J.: A new technique for the assay of infectivity of human adenovirus DNA. Virology 52 (1973) 456–467.

Gregoriadis, G. and Allison, A.C. (Eds.): Liposomes in Biological Systems. Wiley, Chichester, 1980.

Helling, R.B., Goodman, H.M. and Boyer, H.W.: Analysis of endonuclease R · *Eco*RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose gel electrophoresis. J. Virol. 14 (1974) 1235–1244.

Huang, R.R.C., Rott, R., Wahn, K.H.-D. and Kohama, T.: The function of the neuraminidase in membrane fusion induced by myxoviruses. Virology 107 (1980) 313–319.

Inler, G., Glew, R. and Schnure, F.: Enzyme loading of erythrocytes. Proc. Natl. Acad. Sci. USA 70 (1973) 2663–2666.

Kingsbury, D.W.: Newcastle disease virus RNA, I. Isolation and preliminary characterization of RNA from virus particles. J. Mol. Biol. 18 (1966) 195–203.

Kislina, O.S., Naroditsky, B.S., Grigoriev, V.B., Klimenko, S.M. and Tikchonenko, T.I.: Efficiency of inclusion of viral DNA condensed with spermine into liposome. Liposomal interactions with cells. Mol. Genet. Mikrobiol. Virol. 2 (1985) 17–21 (in Russian).

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680–685.

Lowry, O.H., Rosebrough, N.I., Farr A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 (1951) 265–275.

Maeda, T. and Ohnishi, S.: Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. FEBS Lett. 122 (1980) 283–287.

Maniatis, T., Jeffrey, A. and Kleid, D.E.: Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. USA 72 (1975) 1184–1188.

Maniatis, T., Fritsch, E.F. and Sambrook, J.: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

Mertz, I. and Berg, P.: Defective simian virus 40 genomes: isolation and growth of individual clones. Virology 62 (1974) 112–124.

Rathleen, D. and Lawren, S.: Efficient infection of monkey cells with SV40 DNA. J. Virol. Methods 5 (1982) 335–341.

Sato, S.B., Kawasaki, P. and Ohnishi, S.I.: Hemolytic activity of influenza virus hemagglutinin glycoproteins activated in mildly acidic environments. Proc. Natl. Acad. Sci. USA 80 (1983) 3153–3157.

Schwendener, R.A., Lagocki, P.A. and Rahman, Y.E.: The effects of charge and size on the interaction of unilamellar liposomes with macrophages. Biochim. Biophys. Acta 772 (1984) 93–101.

Shen, D.-F., Huang, A. and Huang, L.: An improved method for covalent attachment of antibody to liposomes. Biochim. Biophys. Acta 689 (1982) 31–37.

Straus, S.E., Wilson, T. and Raskas, H.J.: Transfection of KB cells by liposomes containing adenovirus type 2 DNA. J. Virol. 39 (1981) 290–294.

Svetashev, V.I. and Vaskovsky, V.E.: A simplified technique for thin-layer microchromatography of lipids. J. Chromatogr. 67 (1972) 376–378.

Szoka, F. and Papahadjopoulos, D.: Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse phase evaporation. Proc. Natl. Acad. Sci. USA 75 (1978) 4194–4198.

Szoka, F., Magnusson, K.E., Wojcieszyn, J., Hou, Y., Derzko, Z. and Jacobson, K.: Use of lectin and polyethylene glycol for fusion of glycolipid-containing liposomes with eukaryotic cells. Proc. Natl. Acad. Sci. USA 78 (1981) 1685–1689.

Szybalska, E.H. and Szybalski, W.: Genetics of human cell lines, IV. DNA-mediated heritable transformation of a biochemical trait. Proc. Natl. Acad. Sci. USA 48 (1962) 2026–2034.

Torchilin, V.P. and Klibanov, A.L.: Immobilization of proteins on liposome surface. Enzyme Microbiol. Technol. 3 (1981) 297–304.

Torchilin, V.P., Omelyanenko, V.G., Klibanov, A.L., Goldmakher, V.S. and Smirnov, V.N.: Incorporation of hydrophilic protein modified with hydrophobic agent into liposome membrane. Biochim. Biophys. Acta 602 (1980) 511–521.

Van der Eb, A.J. and Graham, F.L.: Assay of transforming activity of tumor virus DNA. Methods Enzymol. 65 (1980) 826–839.

White, J., Helenius, A. and Gething, M.-J.: Hemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. Nature 300 (1982) 658–659.

Wilson, I.A., Skehel, J.J. and Wiley, D.C.: Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3Å resolution. Nature 289 (1981) 366–373.

Wong, T.-K., Nicolau, C. and Hofschneider, P.H.: Appearance of β-lactamase activity in animal cells upon liposome-mediated gene transfer. Gene 10 (1980) 87–94.

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